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p53 and Cancer-associated Fibroblasts: Implications for Cancer Therapy and Drug Resistance

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p53 and Cancer-associated Fibroblasts: Implications for Cancer Therapy and Drug Resistance

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Success is not final,

failure is not fatal:

it is the courage to

continue that counts.

- Winston Churchill

ABSTRACT

Drug resistance remains as a major problem and a daunting challenge to successful anti-cancer treatment. Cancer cells are masters of adaptation. Novel drugs that attack cancer in new ways and target key drivers of cancer cell growth are, therefore, urgently needed. At the same time, the more genetically stable cells of the tumor microenvironment are being recognized as important players in tumor development that can confer resistance to anti-cancer drugs. Increasing attention is being paid to the tumor microenvironment, including cancer-associated fibroblasts (CAFs). Furthermore, the tumor suppressor gene TP53, which codes for the p53 protein, plays an important role in tumor suppression and cellular stress responses to DNA damage and anti-cancer agents. For this reason, the TP53 gene is frequently mutated in cancer. In the absence of mutations, the p53 protein is often downregulated or inactivated through other mechanisms.

This thesis aims to elucidate pathways or mechanisms that contribute to CAF-mediated drug resistance in prostate cancer. The thesis is also focused on investigating a combinatorial therapeutic strategy to improve the effectiveness of the mutant p53-reactivating compound APR-246.

The studies in **paper I** and **II** revealed that CAFs can enhance cell survival, and affect the sensitivity of prostate cancer cells carrying wild type p53 to chemotherapeutic drugs through different mechanisms. In the **paper I**, we showed that glutathione, produced by CAFs, protects cancer cells from drug-induced oxidative stress and DNA damage, as it also decreases drug accumulation. In the **paper II**, we demonstrated that IL-6, one of the soluble factors secreted by CAFs, attenuates the drug-induced p53 response through STAT3 and MDM2. The increased resistance to chemotherapeutic drugs is likely to be a result of the combined effect of glutathione and cytokines like IL-6. In the **paper III**, we found that inhibition of the efflux pump MRP1 enhances APR-246-induced mutant p53 cancer cell death both *in vitro* and *in vivo*. This study also highlighted the impact of the cellular redox status and glutathione content on cancer cell survival and anti-tumor activity of APR-246.

In conclusion, pathways or factors that potentially contribute to drug resistance have been the focus of this thesis. Manipulation of these targets in combination with traditional therapies may lead to a more efficient cancer therapy. This thesis also highlights CAFs as a potential target for anti-stromal therapies in prostate cancer.

LIST OF SCIENTIFIC PAPERS

I. **Human cancer-associated fibroblasts enhance glutathione levels and antagonize drug-induced prostate cancer cell death.**
Emarndeena H. Cheteh, Martin Augsten, Helene Rundqvist, Julie Bianchi, Victoria Sarne, Lars Egevad, Vladimir JV Bykov, Arne Östman, Klas G Wiman.
Cell Death Dis. 2017 Jun 1;8(6):e2848

II. **Interleukin-6 derived from cancer-associated fibroblasts attenuates the p53 response to doxorubicin in prostate cancer cells.**
Emarndeena H. Cheteh, Victoria Sarne, Sophia Ceder, Julie Bianchi, Martin Augsten, Helene Rundqvist, Lars Egevad, Arne Östman, Klas G Wiman.
Cell Death Discov. 2020 Jun 2;6:42

III. **A thiol-bound drug reservoir enhances APR-246-induced mutant p53 tumor cell death.**
Sophia Ceder, Sofi E. Eriksson, **Emarndeena H. Cheteh**, Swati Dawar, Mariana Corrales Benitez, Vladimir Bykov, Kenji M. Fujihara, Lars Abrahmsen, Nicholas J. Clemons, Klas G. Wiman.
EMBO Mol Med. Under revision

CONTENTS

LIST OF ABBREVIATIONS

^{14}C-APR-246	^{14}C labelled APR-246
5-FU	5-fluorouracil
ABC	ATP binding cassette
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BCR-ABL	Breakpoint cluster region gene fused with ABL gene
BCRP	Breast cancer resistance protein
CAFs	Cancer-associated fibroblasts
CCL2	C-C motif chemokine ligand 2
CCL5	C-C motif chemokine ligand 5
CDKN1A	Cyclin-dependent kinase inhibitor 1
CML	Chronic myeloid leukemia
CNTF	Ciliary neurotrophic factor
CTGF	Connective tissue growth factor
CXCL12	C-X-C motif chemokine ligand 12 or SDF1
CXCL14	C-X-C motif chemokine ligand 14
Da	Dalton
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
EndMT	Endothelial-mesenchymal transition
FAP	Fibroblast-activated protein
FGF2	Fibroblast growth factor 2
FSP1	Fibroblast-specific protein 1
GCL	Glutamate cysteine ligase
GGT	γ-glutamyl transpeptidase
gp130	Glycoprotein 130

GPX	Glutathione peroxidase
GR	Glutathione reductase
GS-MQ	MQ bound to glutathione
GSH	Reduced glutathione
GSS	Glutathione synthetase
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HPV	Human papilloma virus
IARC	The International Agency for Research on Cancer
IGF	Insulin growth factor
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
IL-11	Interleukin-11
JAK	Janus kinase
kDa	Kilodalton
LIF	Leukemia inhibitory factor
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia-1
MDM2	Murine double minute 2
MDR1	Multidrug resistance 1
MDS	Myelodysplastic syndrome
MMP	Matrix metalloproteinase
MQ	Methylene quinuclidinone
mRNA	Messenger RNA
MRP1	Multidrug resistance-associated protein 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NK cell	Natural killer cell
Nrf2	Nuclear factor erythroid 2-related factor 2
$O_2^{-\bullet}$	Superoxide anion
$OH^{\bullet}$	Hydroxyl radical

OPG	Osteoprotegerin
OSM	Oncostatin M
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
pg/ml	Picogram per milliliter
PI3K	Phosphatidylinositol 3-kinase
PRDX	Peroxiredoxin
PRIMA-1	p53 Reactivation and Induction of Massive Apoptosis
PRIMA-1Met	Methylated PRIMA-1
PTEN	Phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDF1	Stromal-cell-derived factor 1
sIL-6R	Soluble interleukin-6 receptor
siRNA	Small interfering RNA or silencing RNA
SLC7A11	Solute carrier family 7 member 11
SOD	Superoxide dismutase
SSZ	Sulfasalazine
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
TNM	Tumor, Node, Metastasis
TP53	Tumor protein 53
Trx	Thioredoxin
TYK2	Tyrosine kinase 2
VEGF	Vascular endothelial growth factor
Wnt	Wingless-related integration site
α-SMA	Alpha-smooth muscle actin

1 INTRODUCTION

1.1 CANCER

Cancer is a major public health problem, as well as a complex and dynamic disease. It is also a common disease, as in 2018 there were about 18 million new cases of cancer reported and 9.6 million deaths worldwide [1]. In fact, cancer is not just one disease but rather a collection of more than 100 different types, with varied characteristics, involving various causes and risk factors, and requiring wide-ranging treatments.

1.1.1 What is cancer and what are its causes?

Cancer starts when cells in our body grow out of control and crowd out normal cells (Figure 1). This can happen at any place in the body and cause damage to organs affected. Continuation of uncontrolled growth of cells results in rapid cell accumulation that with time gives rise to tumor lumps or solid tumors. However, not all lumps are cancer. Unlike cancerous or malignant tumors, a benign tumor grows locally without spreading to other parts of the body and can be left untreated. A malignant tumor, on the other hand, has the potential to invade nearby tissue and later metastasize, i.e. dissociate and spread, to distant areas of the body through the blood or the lymphatic system. Meanwhile, there are also cancers that don't form lumps, for example leukemia.

Cells become cancer cells largely because of errors or mutations in the cells' DNA – their genetic "blueprint". Mistakes and gene mutations may happen all the time as cells divide, but cells are programmed to detect the alterations and repair them. In more severe cases, where damages cannot be repaired, the cells will be signaled for programmed cell death. Only cells with mutations that manage to escape and survive may give rise to cancer, particularly if the mutation affects a gene that regulates cell growth and division. Regardless, it takes more than one mutation in a cell for cancer to occur, and because the numbers of gene mutations increase and build up over time, the risk of getting cancer is higher as we get older.

Some of the abnormal changes in cells' DNA may be passed from generation to generation, when an inherited gene mutation is present in the egg or sperm, while other mutations may be caused by environmental factors. Acquired mutations are thought to cause a majority of all cancer cases, and only about 5-10% are the result of inherited gene mutations [2].

The risk of developing certain kinds of cancers can be increased by lifestyle or specific exposures. Cancer-causing, or carcinogenic, substances have been listed by The International Agency for Research on Cancer (IARC) [3], for different groups depending on the potential of these chemicals in causing cancer.

1.1.2 Oncogenes and tumor suppressor genes

Two main types of genes that play a key role in cancer induction are tumor suppressor genes and oncogenes.

Tumor suppressor genes prevent a cell from undergoing uncontrolled division and abnormal growth. They function to halt the cell cycle if there is a problem until certain events are

completed. They also induce the necessary DNA repair processes or cell death upon cellular damage or mistakes. Tumor suppressor genes including TP53, PTEN (involved in counteracting the action of the oncogenes PI3K and Akt) and Rb (responsible for the G1 checkpoint and blocking S-phase entry) are frequently lost or inactivated in many cancers [4]. In addition, suppression of tumor suppressor genes occurs much more frequently than activation of oncogenes. TP53 will be further discussed in chapter 1.8 THE TUMOR SUPPRESSOR p53.

Proto-oncogenes, which normally regulate cell growth and division, are transformed to oncogenes by mutations and become permanently activated. As a result, cells grow and divide rapidly through growth-promoting signals. Oncogenes are generally activated by point mutation, chromosomal rearrangement, which allows one gene to activate another, or by gene duplication and amplification. Most frequently encountered oncogenes in cancers are for instance Ras and Myc gene family, and growth factor receptors [4].

1.1.3 Cancer types

Cancer can be divided into four major subtypes according to the type of cells that form them[5]. These major classifications are:

I. Carcinomas

II. Sarcomas

III. Hematopoietic malignancies

IV. Neuroectodermal malignancies

All cell types in the body can give rise to cancer but the majority of human tumors are carcinomas, which arise from epithelial tissues (Figure 1). Epithelial cells are those that line the walls of cavities of internal organs, the outer surfaces of organs or cover the surface of the body, i.e. the skin. Carcinomas in turn fall into two major categories, which are squamous cell carcinomas and adenocarcinomas. Squamous cell carcinomas are generated from epithelial cells that form protective cell layers, while epithelial cells that secrete substances into the ducts or cavities give rise to adenocarcinomas.

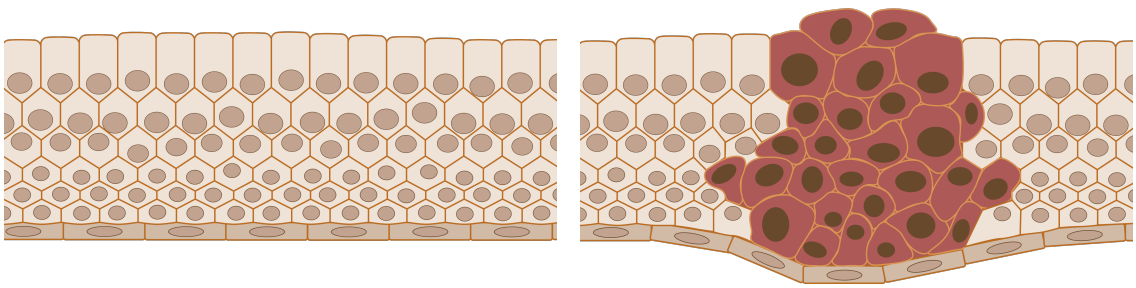

Figure 1. A normal tissue with epithelial cells grown in an orderly way vs. carcinoma with tumor cells dividing rapidly

(Figure adapted from https://www.cancerresearchuk.org/about-cancer/what-is-cancer)

The four most common cancers occurring worldwide are lung, breast, colorectal and prostate cancer [6], while lung, colorectal, stomach and liver cancer [7] are the most common causes of cancer death. In Sweden, skin cancer is also one of the most common cancers [8].

The rest of malignant tumors are derived from nonepithelial tissues. Various cells of connective tissues, such as fibroblasts, adipocytes (fat cells), osteoblasts (bone-forming cells), myocytes (muscle cells) and endothelial cells (lining of blood vessels and lymph vessels) can give rise to sarcoma.

Hematopoietic malignancies are caused by different cells that constitute the blood-forming tissues, including the bone marrow and the lymphatic system. Leukemia (cancer of white blood cells) and lymphomas are examples of this type of cancer.

Neuroectodermal malignancies are derived from various cells of the nervous system. Glioblastomas (develop from star-shaped glial cells), retinoblastomas (develop from the immature cells of a retina) and neuroblastomas (develop from immature nerve cells) can be included in this group.

Some types of tumors, such as melanomas that are derived from melanocytes (melanin-producing cells), do not fit into any of these four groups.

1.2 HALLMARKS OF CANCER

As previously described, mutations in the DNA are the underlying cause of normal cells becoming cancerous. These genetic alterations cause disruption in regulatory circuits that control normal cell function and homeostasis, leading cells to turn on genes that are normally turned off or silence genes that should be turned on. Even so, development of cancers is a multistep process with some common features. Hanahan and Weinberg [9] have summarized six distinctive biological capabilities that cells acquire during transformation to malignancy:

Sustaining proliferative signaling – Cancer cells are self-sufficient in growth signals and do not need stimulation from external signals to multiply. They can do so in a number of ways. They may, for instance, produce growth factor ligands themselves or increase the levels of receptors at the cell surface.

Evading growth suppressors – Cancer cells are also insensitive to anti-growth signals. They, for instance, inactivate tumor suppressor genes or disrupt the growth suppression pathway.

Resisting cell death – Cancer cells learn how to avoid and escape the process of programmed cell death apoptosis, by downregulating expression of proapoptotic regulators or increasing antiapoptotic or survival factors.

Enabling replicative immortality – Cancer cells are capable of multiplying without any limit, i.e. they are immortalized, by turning on telomerase.

Inducing angiogenesis – Tumors form new blood vessels from existing ones in order to provide themselves with sufficient supply of nutrients and oxygen, as well to remove metabolic wastes and carbon dioxide.

Activating invasion and metastasis – Cancer cells move out of the primary tumor mass, invade surrounding tissues and travel to distant sites, where they may found new tumor colonies.

The acquisition of these hallmark capabilities is made possible by **genomic instability** in cancer cells that generates random mutations, and **tumor-promoting inflammation** [10]. Cancer has been considered as an inflammation that never really resolves. Persistent inflammatory response contributes to hallmark capabilities by supplying bioactive molecules to the tumor microenvironment.

Later, two more capabilities are added as emerging hallmarks [10]:

Reprogramming of energy metabolism – Cancer cells alter the metabolic pathways to satisfy their increased needs for energy and building materials.

Evading immune destruction – Tumor cells develop strategies to bypass the surveillance of the immune system, which plays a major role in eliminating them.

In addition to cancer cells, a repertoire of recruited non-transformed cells also contributes to the development of certain hallmark capabilities. These recruited non-transformed cells form a permissive microenvironment, called the 'tumor microenvironment', which turns out to be crucial for the progression and maintenance of the cancerous cells they surround via a tight and dynamic interaction between the two.

1.3 THE TUMOR MICROENVIRONMENT

Aside from tumor cells, the tumor microenvironment is comprised of cancer stem cells, blood vessels including endothelial cells and pericytes, immune inflammatory cells and other stromal cells (connective tissue cells) such as fibroblasts (Figure 2). Unlike cancer and cancer stem cells, stromal cells in the tumorigenic microenvironment are genetically stable. However, they are recruited and reeducated by cancer cells in order to support cancer cells in various ways, and may carry epigenetic changes. Hence, they display altered gene expressions when compared to their non-tumoral counterpart, despite their untransformed status.

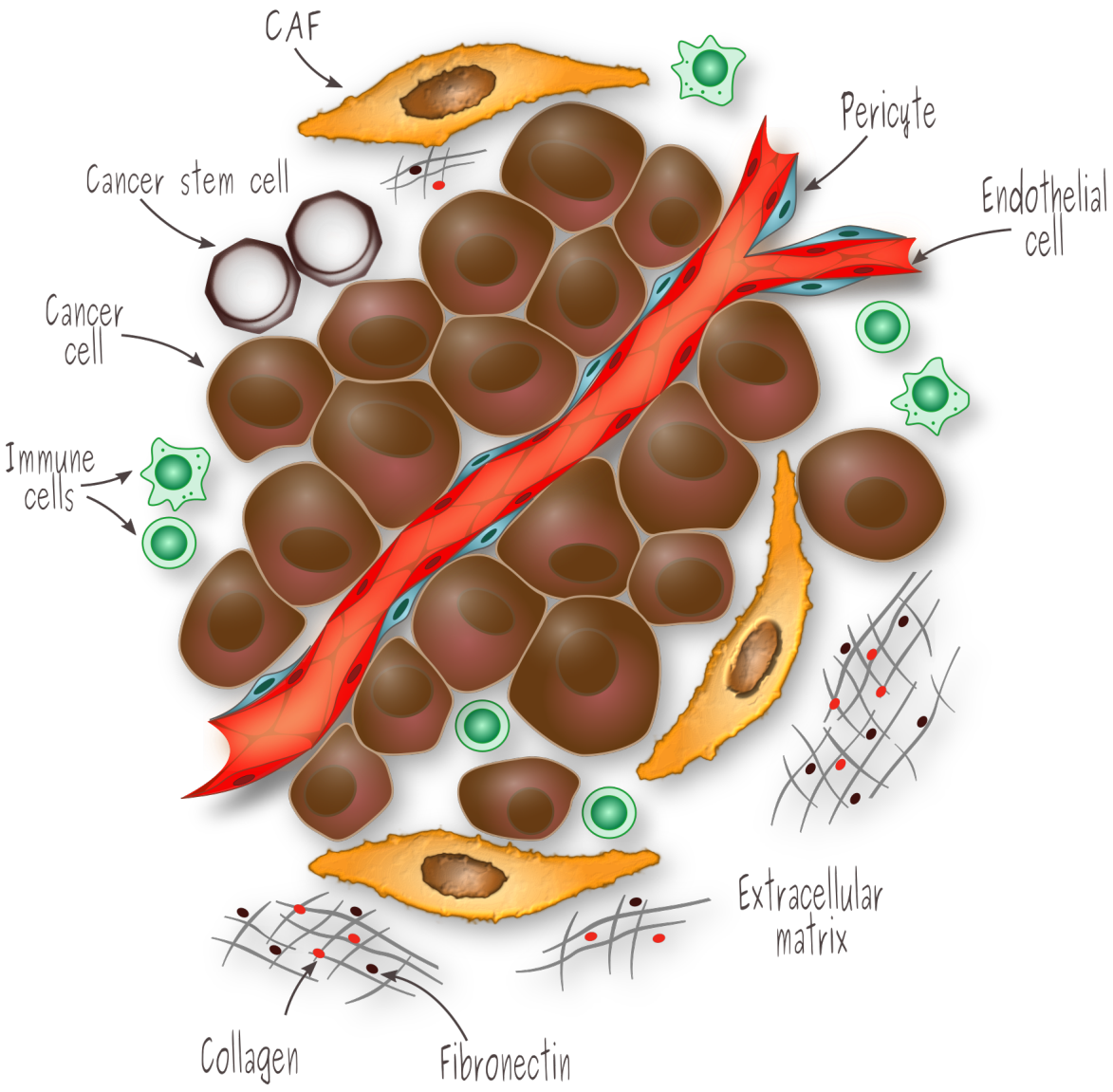

Figure 2. Tumor cells and the tumor microenvironment

1.3.1 Cancer stem cells

Cancer cells within a tumor have, in recent years, been considered as heterogeneous cell populations. This could in part be a result of the hyperproliferation combined with enhanced genetic instability that leads to diverse clonal subpopulations. A solid tumor displays a great degree of heterogeneity of tumor cells with various states of differentiation, proliferation rates, migratory and invasive capacity, or other features [11,12]. More and more evidence points towards the existence of cancer stem cells or that a fraction of cells within most tumors has acquired properties of stem cells. With self-renewal ability, similar to that of normal tissue stem cells, cancer stem cells are able to maintain tumor growth or seed new tumors. They are as well capable in giving rise to a range of distinct cancer cell types within a tumor [13].

1.3.2 Endothelial cells

A layer of endothelial cells forms the inner lining of blood vessels. Activation of normal endothelial cells by cancer cells causes them to transform into tumor-associated endothelial cells with ability to construct new blood vessels by angiogenesis. The balance between pro- and anti-angiogenic factors that regulates vascularization in normal tissues is disrupted. Instead, the pro-angiogenic stimuli are taking over in this angiogenic switch. One of the prominent key regulators for blood vessel sprouting is vascular endothelial growth factor (VEGF), which is highly expressed in most tumors. The newly formed vasculature serves as a supply line for nutrients, oxygen and other solutes from the bloodstream to cancer cells. It is also used for removing waste products [14].

The tumor blood vessels also differ from their normal counterpart, as they tend to be leaky, fragile and highly irregular. Blood flow also tends to be low and abnormal. High proliferating cancer cell density around the tumor vessels gives rise to compression of the blood vessels and increased microvascular pressure [14]. This high microvascular pressure, together with poor tumor vessel quality, low blood flow, poor perfusion (blood gets to a tissue) and inadequate lymphatic drainage of excess fluid leads to an elevated interstitial fluid pressure in solid tumors [15]. This results in reduced fluid movement through the interstitium (fluid filled space between cells), and becomes an obstacle in cancer therapy.

1.3.3 Pericytes

Pericytes are vascular smooth muscle cells or mural cells that wrap around the endothelial cells at the surface of the blood vessels. Both pericytes and endothelial cells collaborate with each other to structure the vascular basement membrane, in order for vessel walls to withstand the pressure of blood flow. They communicate by direct cell-cell contact or through paracrine signaling pathways, which allows the exchange of small molecules. Unlike normal pericytes, the tumor pericytes appear to be loosely attached to the vasculature and exhibit abnormal pericyte behavior or expression profile. In some tumors, a larger number of pericytes express alpha-smooth muscle actin (α-SMA), which is normally expressed by cells of the smooth-muscle lineages [16,17].

The recruitment of pericytes into tumors is largely dependent on the platelet-derived growth factor (PDGF) signaling, where tumor pericytes express PDGF receptor-β and PDGF-β ligand is produced by endothelial cells. By perturbing the expression of PDGFR-β or its ligand results in reduced pericyte coverage of tumor vessels, which in turn leads to more disordered and defective vasculature [16,17]. In consequence, tumor vessels without pericytes appear to be more vulnerable and may be a good target for anti-angiogenic therapy.

Tumor pericytes, despite having structural and behavioral abnormalities, still contribute to tumor angiogenesis, provide functions necessary for vessel maintenance and support endothelial cell survival.

1.3.4 Immune inflammatory cells

In normal wound healing and defenses against inflammation or infection, immune cells appear transiently and disappear when the target intruder is destroyed. Tumors, however,

have been associated with chronic inflammation or portrayed as wounds that never heal. Diverse types of cells of the immune system are, therefore, found to infiltrate tumor tissues. Surprisingly, many of these tumor-infiltrating inflammatory cells, including macrophages, mast cells, neutrophils, T and B lymphocytes have tumor-promoting effects rather than serving as effectors of tumor-antagonizing actions. These cells have been shown to stimulate cancer cell proliferation, induce tumor angiogenesis, and support metastasis and tissue invasion. Several signaling molecules released by the immune inflammatory cells are also linked to their tumor-promoting actions. These include epidermal growth factor (EGF), VEGF, fibroblast growth factor 2 (FGF2) and other inflammatory associated chemokines and cytokines [10].

In addition to fully differentiated immune cells, partially differentiated myeloid progenitor cells can also be found in tumors. These recruited progenitor cells have been shown to support tumor progression and allow cancer cells to evade immune destruction, for instance by suppressing cytotoxic T lymphocyte and NK cell activity [10].

1.3.5 Fibroblasts

Fibroblasts are spindle-shaped cells and are the most common cell types of connective tissue. They produce collagen and components that make up the extracellular matrix (ECM) (Figure 2), while also supporting the structure of tissues and organs. Fibroblasts also play a critical role in wound healing, e.g. when tissues are injured, they are activated and start expressing α-SMA as well as generate contraction to facilitate wound closure. Once the healing process is completed, these activated fibroblasts are removed by apoptosis [18].

As previously mentioned, tumors have been described as wounds that never heal. Normal fibroblasts can be reprogrammed and turn into cancer-associated fibroblasts (CAFs) by a variety of chemical signals from cancer cells and remain constitutively active. However, their biological roles and properties differ markedly from that of normal fibroblasts.

CAFs will be discussed in more detail in chapter 1.4.

1.4 CANCER-ASSOCIATED FIBROBLASTS

Most solid tumors contain activated or cancer-associated fibroblasts (CAFs) and their abundance varies between different cancer types. Prostate and breast cancer for instance are known to contain high numbers of CAFs. CAFs are also the most abundant cell type in the tumor stroma [19].

1.4.1 Phenotypes and tumor promoting effects of CAFs

In contrast to normal fibroblasts, CAFs can be characterized with phenotypes such as more rapid proliferation rate, enhanced collagen production and secretion of growth factors. They are either found to reside at the edge of tumors or disperse through out the tumor mass [20]. Apart from these phenotypes, they are also a potent supporter of carcinogenesis. They have the capability to promote the initiation of epithelial tumor formation, tumor growth and progression, angiogenesis, metastasis, inflammation as well as immunosuppression [20-22].

CAFs can support tumorigenesis by regulating immune cells and contributing to immune escape of tumors [22]. Another feature of CAFs is the ability to remodel the ECM. The changes in the composition of the ECM influence the stiffness of the tissue, where the tumor ECM is denser and stiffer than normal tissue. This has an effect on tumorigenic behaviors and on how tumors respond to therapy. The dense and rigid ECM, due to increased production of collagen and other ECM molecules by CAFs, can shield cancer cells from therapeutic agents. CAFs contribute not only to the ECM modification but also regulate ECM degradation, which then facilitates tumor growth, invasion and migration [19,21].

CAFs also display altered gene expressions compared to their non-tumoral counterpart. Many of these genes encode receptors or secreted proteins, which are cytokines, growth factors and signaling molecules [23]. Several of these proteins are well known for their involvement in tumor-stroma cell interaction. CAF-derived chemokine such as stromal-cell-derived factor 1 (SDF1), also known as CXCL12, enhance cancer cell proliferation and induce angiogenesis. This chemokine has also been reported to augment the proliferation, migration, and invasion in breast cancer. Overexpression of CAF-derived growth factors, such as transforming growth factor beta (TGF-β) and hepatocyte growth factor (HGF), have been shown to influence the tumor growth and progression [20,23,24]. By expressing members of the matrix metalloproteinase (MMP) family, CAFs regulate the degradation of the ECM [19].

Furthermore, CAFs have also been shown to affect sensitivity of tumor cells to chemotherapy and mediate drug resistance, for instance through HGF secretion [20]. CAF-mediated resistance will be further discussed in section 1.10 CANCER DRUG RESISTANCE.

1.4.2 Origin of CAFs

Numerous reports suggest that CAFs can be derived from multiple origins (Figure 3), making up a heterogeneous population of cells. Some of them are local fibroblasts, altered after continuous exposure to cancer cells. Mesenchymal stem cells (multipotent stromal cells) in the vicinity of tumors can differentiate into CAFs. Another possible source of CAFs is recruited smooth muscle cells, which generally express high levels of α-SMA. Cells of epithelial origin also contribute to the CAF population, as they undergo an epithelial-to-mesenchymal transition (EMT). These cells exhibit fibroblast morphology and express at the same time keratin that is only present in epithelial cells. Other studies have demonstrated that endothelial cells can give rise to CAF-like cells, by undergoing endothelial-mesenchymal transition (EndMT) [19,20,24]. These cells often express VEGF. In addition, growth factors, such as TGF-β and PDGF, have been shown to be key mediators in activating and regulating CAFs [25].

In the past few years, a series of studies has demonstrated that different subtypes of CAFs, most likely with different cell-of-origin, differ in functions, confirming the existence of functional heterogeneity among CAFs [25-30]. The breadth of CAF functions, as well as existence of several CAF subpopulations, may pose a challenge in targeting CAFs for clinical benefit.

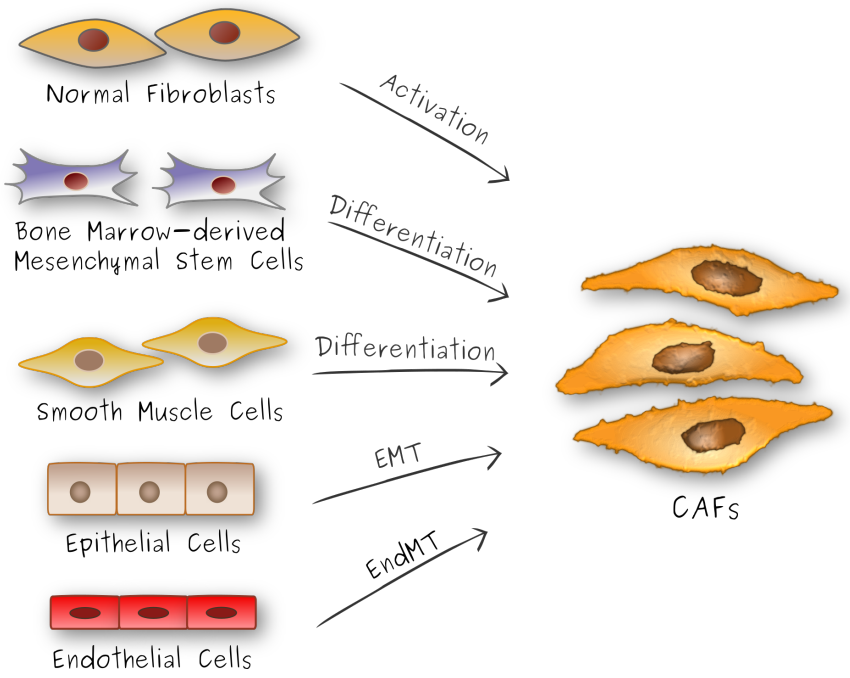

Figure 3. Origin of CAFs

1.4.3 Common CAF markers

The heterogeneity found within the fibroblasts, combined with the presence of multiple subpopulations, make it difficult to identify CAFs based on expression of specific markers. However, they are frequently described to be upregulated in α-SMA, vimentin (a marker of mesenchymal cells), fibroblast-specific protein 1 (FSP1), fibroblast-activated protein (FAP) and PDGFR-β, while caveolin-1 (a protein of caveolae, lipid raft) and laminin (largely expressed in epithelial basement membranes) are reported to be downregulated in CAFs [19-21,24]. These markers still do not mark all or are unique to CAFs.

1.5 SECRETED FACTORS AND CYTOKINES IN CANCER

As mentioned earlier, cancer cells use different tactics to promote their own growth and survival. In order to do so, they communicate with each other and with its healthy neighbor cells through signaling pathways mediated by secreted low-molecular-weight factors and signaling molecules. These secreted factors and signaling molecules, also called cytokines, play an important role in this communication, and they are rapidly synthesized by the producer cells. These producer cells are often adjacent to target cells, which facilitates the binding of the signaling molecules to specific surface receptors on the target cells. Cancer cells, for instance, interact with nearby cells in a paracrine manner by releasing the secretory factors and cytokines into the tumor microenvironment, which consequently stimulates non-cancerous proximal cells to produce signaling molecules as a response. This complex

network of interaction between tumor cells and their microenvironment results in dynamic feedback loops of stimuli responses. However, this interaction is not only limited to target cells in the neighborhood, as some signaling molecules can bind to distant target cells, acting in an endocrine fashion. Tumor cells also secrete soluble factors and cytokines to influence themselves and exert their actions in an autocrine manner.

The tumor microenvironment often serves as a reservoir of growth factors and cytokines, each of which may affect cancer cells in several ways. The signaling molecules can directly stimulate the proliferation, angiogenesis, and migration, and reduce drug sensitivity of the tumor cells. They can indirectly affect the tumor microenvironment and also alter the production of other cytokines to the benefit of cancer cells. Hence, this bidirectional crosstalk between cancer cells and its useful neighbor through signaling molecules assure both development and maintenance of the tumor itself and of tumor-supportive properties of the microenvironment.

CAFs secrete a variety of soluble factors with protumorigenic activities. Some of the known secreted factors are further summarized in Table 1 [19,20,24,31,32]:

Table 1. A list of secreted factors derived from CAFs and their potential effects on malignant cells

CAF-derived factors	Effects on malignant cells
C-C motif chemokine ligand 5 (CCL5)	⇧ Metastasis
Connective tissue growth factor (CTGF)	⇧ Angiogenesis ⇧ Invasion
C-X-C motif chemokine ligand 14 (CXCL14)	⇧ Proliferation ⇧ Angiogenesis
Epidermal growth factor (EGF)	⇧ Proliferation
Fibroblast growth factor (FGF)	⇧ Angiogenesis
Hepatocyte growth factor (HGF)	⇧ Proliferation ⇧ Invasion
Interleukin-6 (IL-6)	⇧ Proliferation ⇧ Invasion/metastasis
Insulin growth factors IGF-1 and IGF-2	⇧ Proliferation ⇧ Metastasis

Matrix metalloproteinase MMP-1, MMP-2 and other members of the MMP family (through ECM remodeling)	⇧ Proliferation ⇧ Migration/invasion/metastasis
Matrix metalloproteinase MMP-13 (through ECM remodeling)	⇧ Angiogenesis
Stromal cell-derived factor 1 (SDF1) or CXCL12	⇧ Proliferation ⇧ Angiogenesis ⇧ Migration/invasion/metastasis
Transforming growth factor beta (TGF-β)	⇧ Proliferation ⇧ Invasion/metastasis
Vascular endothelial growth factor (VEGF)	⇧ Angiogenesis
Wnt2 and other members of the Wnt family	⇧ Proliferation ⇧ Invasion

IL-6 will be discussed in detail in the next chapter.

1.6 INTERLEUKIN-6

Interleukins were initially thought to be expressed exclusively by leukocytes but were later found to be produced by other cells as well. Interleukin-6 (IL-6), produced for instance by monocytes, macrophages, T cells, mast cells, keratinocytes, fibroblasts and endothelial cells, is a cytokine with a molecular mass of 21-28 kDa [33,34]. It is involved in the host response to injury or infection, immune response and inflammation, and it signals through the gp130/JAK/STAT pathway.

1.6.1 IL-6 biological functions

IL-6, a pleiotropic (multifunctional) cytokine, plays an essential role in host defense as it has wide range of immune and hematopoietic activities, and it regulates the acute-phase response triggered by infections or injuries. IL-6 is an important inducer of the acute phase response in the liver and it also contributes to the body's defense by inducing fever [33]. In addition to its role in the acute phase response, it has the ability to induce differentiation of B cells [35], and proliferation and activation of T cells. Evidence has also shown that IL-6 is capable of inducing entry of hematopoietic stem cells into the cell cycle for proliferation and differentiation [33].

The IL-6 levels in the blood under normal circumstances are around 1-2 pg/ml [36], but can be elevated in patients with infectious diseases and inflammation [37,38].

1.6.2 IL-6 signaling

Classical IL-6 signaling requires IL-6 binding to its non-signaling α-receptor subunit, IL-6R, on the plasma membrane. This ligand binding leads to recruitment of the signal-transducing receptor subunit gp130 to the receptor complex. This then induces gp130 homodimerization, which leads to the activation of Janus kinases (JAKs) and transcription factor of the STAT family [34] (Figure 4). While expression of IL-6R is limited to hepatocytes and subsets of leukocytes [39], gp130 is found in almost all organs [40]. IL-6 can also trigger signal transduction via extracellular secretory soluble receptor (sIL-6R), which forms a complex with gp130 expressed by any cell. The receptor subunit gp130 is also shared by the receptors of other IL-6 family of cytokines, including IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM) and ciliary neurotrophic factor (CNTF) [34].

The Janus kinases JAK1, JAK2 and TYK2 become activated after the assembly of receptor complexes, and subsequently phosphorylate gp130 at specific tyrosine residues within the cytoplasmic part. These phosphotyrosine residues create docking sites for STAT factors STAT1 and STAT3, which in turn become phosphorylated and activated. Recruitment and phosphorylation of STATs at specific tyrosine residues (STAT1: tyrosine 701 and STAT3: tyrosine 705) stimulates them to form dimers, translocate into the nucleus, and bind to enhancer elements of target genes. Consequently, this leads to transcriptional activation of the target genes [34] (Figure 4).

IL-6 may also activate mitogen-activated protein kinase (MAPK) via JAK, which results in enhanced cell growth, proliferation and mitosis. Another signaling pathway that is induced by IL-6 and JAK activation is the phosphatidylinositol 3-kinase/Akt kinase (PI3K/Akt) pathway. JAK-dependent PI3K activity leads to anti-apoptosis signaling and increased cell survival [41].

1.6.3 Role of IL-6 in cancer

IL-6, one of the major cytokines in the tumor microenvironment, and is often found in high concentrations as a result of overproduction by not only non-cancerous cells of the tumor microenvironment but by malignant cells as well. In cancer, IL-6 acts as a pro-inflammatory cytokine and supports tumorigenesis. Both autocrine secretion of IL-6 by cancer cells and paracrine secretion by the tumor microenvironment are known to contribute to tumor proliferation, metastasis, angiogenesis and therapeutic resistance in many cancers. In the case of prostate cancer, inhibition of IL-6 increases the sensitivity of prostate tumor cells to anti-cancer therapies, and high serum IL-6 levels have been reported to correlate with poor therapeutic response [42].

Elevated IL-6 levels are often detected in affected tissues or serum from patients diagnosed with cancer. As high as 6-12 pg/ml of IL-6 has been measured in the serum from prostate cancer patients [43-45]. Moreover, IL-6 levels have been reported to be higher in prostate cancer patients with metastatic disease (bone or lymph node metastases) than in patients with earlier stages of the disease or with localized prostate cancer as well as healthy individuals. Patients

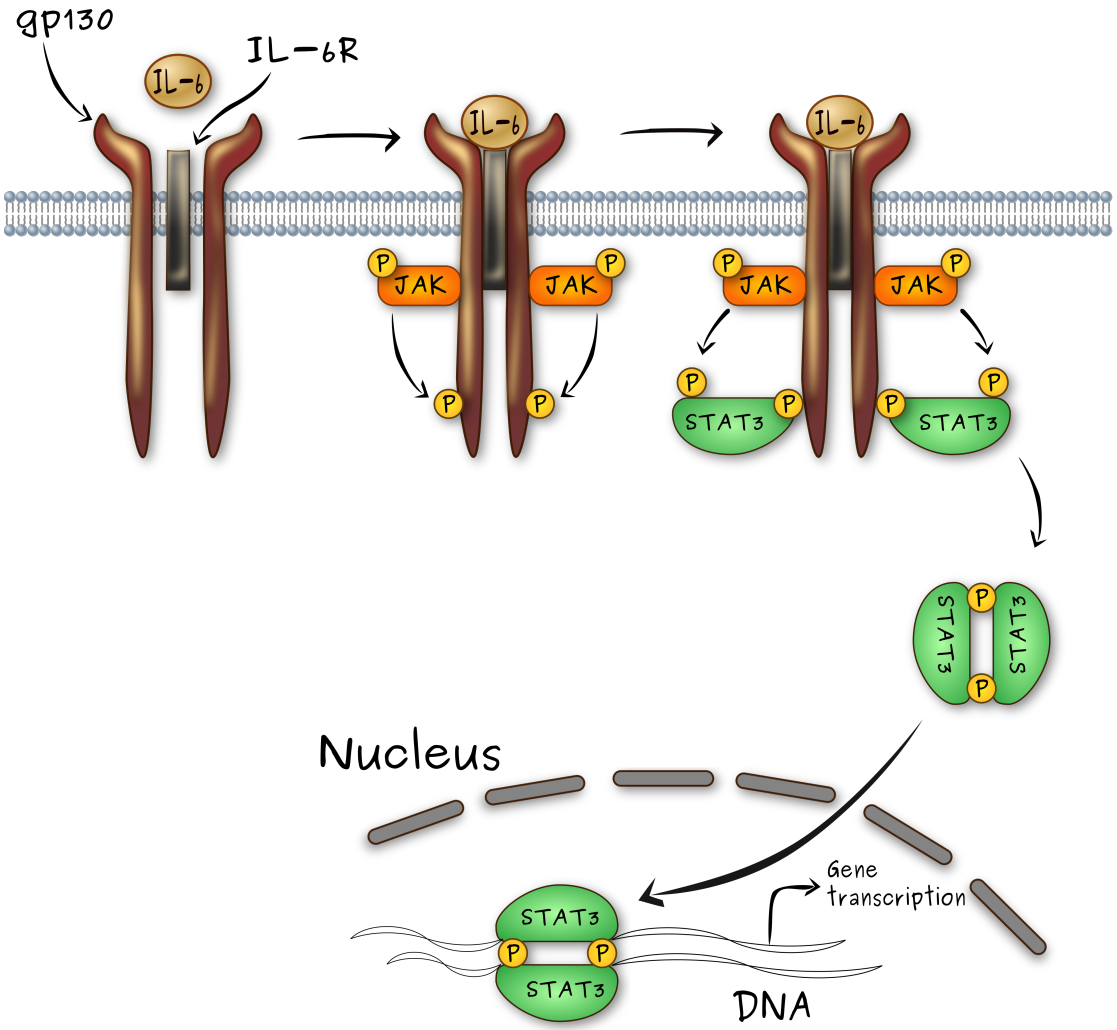

Figure 4. IL-6 signaling through the gp130/JAK/STAT3 pathway

P = Phosphorylation

with castration-resistant prostate cancer also had elevated levels of IL-6 compared to the hormone sensitive group [41].

Prostate cancer cells are normally dependent on androgens, especially testosterone. Patients with advanced disease are submitted to hormonal castration, also called androgen suppression therapy. However, the androgen receptor can be activated by other cytokines and growth factors, including IL-6, in the absence of androgen. Castration-resistant prostate cancer has therefore been observed after hormonal castration, when the tumor shows decreased sensitivity to the treatment with androgen deprivation. This is a problem as it is an incurable stage of the disease. Eventually patients develop metastases and most patients die from recurrent castration-resistant prostate cancer [41].

Increased serum levels of IL-6 have also been associated with an adverse prognosis in patients with different types of cancer, including prostate cancer [46]. Shorter survival in

prostate cancer patients has been shown to correlate with increased IL-6 levels and aggressive disease [41].

Furthermore, STAT3 activation mediated by IL-6 can regulate the process of apoptosis. STAT3-dependent upregulation of anti-apoptotic regulators, such as Bcl-2, Mcl-1, Bcl-xL and survivin, helps cancer cells to resist cell death. The Bcl-2 proteins Bcl-2 and Mcl-1 interact for instance with Bax and prevent the induction of apoptosis [47].

1.7 GLUTATHIONE

Glutathione is a tripeptide of the amino acids glutamic acid-cysteine-glycine (Figure 5) with a molecular weight of approximately 307 Da [48]. Stromal cells are able to secrete glutathione and the amino acid cysteine, to neighboring cancer cells and promote cancer cell survival [49,50].

Glutathione plays a central role in the detoxification of xenobiotics (exogenous and foreign substances for the body) and endogenously generated reactive metabolites. It is the major antioxidant produced by the cells and it protects our body from free radicals, which are highly reactive forms of oxygen or nitrogen with unpaired electrons and readily react with other molecules, causing damage to cells and its DNA. By directly binding to harmful oxidative molecules, glutathione neutralizes a wide range of oxidants. Glutathione is also used as a cofactor by several antioxidant enzymes [51].

1.7.1 Glutathione biosynthesis

Glutathione synthesis involves two enzymatic steps, which includes glutamate cysteine ligase (GCL) in the first step and glutathione synthetase (GSS) in the second (Figure 5). The rate of glutathione synthesis is mostly determined at the first step by two factors: cysteine availability and GCL activity [52]. Cysteine is normally derived from protein breakdown or by conversion of methionine through the transsulfuration pathway [48,52]. Furthermore, both cysteine and its oxidized form cystine can be imported via different membrane transporters. Cystine that occurs mainly in a more oxidative environment is imported by the cystine/glutamate antiporter xCT (SLC7A11). The imported cystine is rapidly reduced to cysteine by the thioredoxin (Trx) system or the glutathione system [48,53] (Figure 5).

Glutathione exists in cellular systems in two states. The free glutathione, which is the reduced form (GSH), makes up the vast majority in the intracellular compartment and is present at millimolar concentrations under normal conditions. Meanwhile, oxidized glutathione (GSSG) is estimated to constitute less than 1% of the total glutathione. 90% of glutathione can be found in the cytosol and nearly 10% in mitochondria [52].

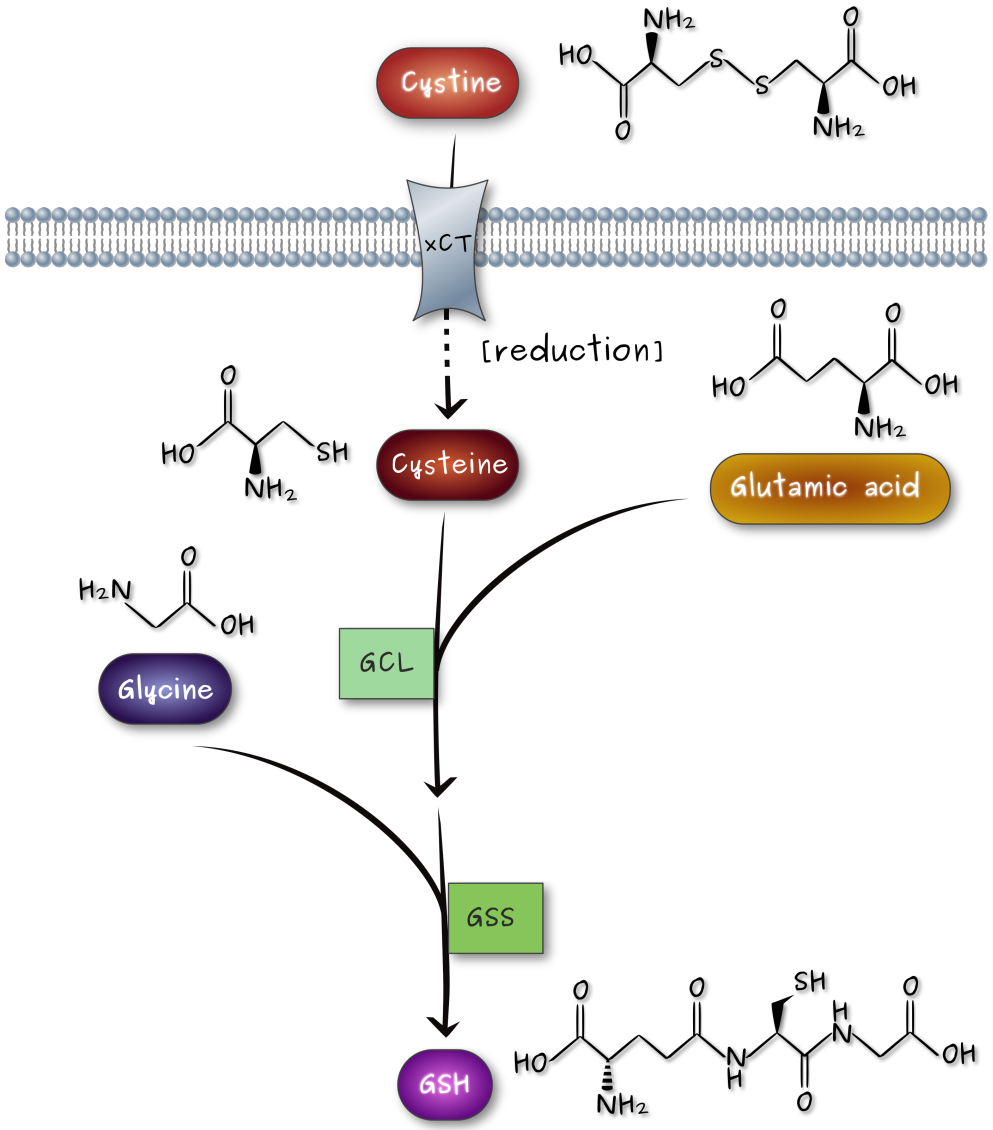

Figure 5. The synthesis of glutathione from its constituent amino acids involving two enzymatic steps

1.7.2 Glutathione functions

Oxidants or reactive oxygen species (ROS) are generated by aerobic cells as products of a normal cellular metabolism and most of them by the mitochondrial respiratory chain. During endogenous metabolic reactions, the reactive metabolites, such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and superoxide anion ($O_2^{-\bullet}$), are produced. Under conditions of cell injury or stress, ROS production is further increased, resulting in an imbalance in cellular redox homeostasis, which can cause severe damage to the cell. To prevent irreversible cell damage, an increase of ROS leads to induction of the cellular protective mechanisms referred to as antioxidant defenses. Antioxidants are induced to protect the cells and their components, including mitochondria and DNA, against the increased levels of oxidant species. Cells have non-enzymatic and enzymatic ways of ROS elimination. Detoxification from ROS can be facilitated by glutathione, a low-molecular weight thiol, or by enzymatic antioxidants such as glutathione reductase (GR), glutathione peroxidase (GPX), peroxiredoxin (PRDX), catalase, and superoxide dismutase (SOD). These antioxidants aim to control the balance between

production and removal of ROS, and thereby restore the redox homeostasis [54,55]. Among the antioxidants involved in the maintenance of the intracellular redox balance, an essential role is played by glutathione. Depletion of glutathione due to excessive antioxidant protection or detoxification may create an imbalance in redox homeostasis. Reactive metabolites, such as H_2O_2, can be metabolized by GPX, and this reaction involves oxidation of GSH to GSSG. In order to limit oxidative damage, GSSG is reduced back to GSH by GR (Figure 6) at the expense of NADPH [52].

Other harmful and toxic substances can be processed by forming conjugates with glutathione. The conjugation occurs either spontaneously or is catalyzed by glutathione-S-transferase (GST) [52]. GST attaches glutathione to the toxins and makes them more water-soluble and ready to be eliminated. Furthermore, glutathione regulates thiolation of proteins, which is a common feature of redox signal transduction and is essential for several biological processes, such as glycolysis, proteolysis, modulation of enzymes, and regulation of protein function [56]. At the same time, glutathione also serves as a source of cysteine. Glutathione levels are also maintained through a salvage pathway, the γ-glutamyl cycle, which involves the release of glutathione from the cell, the breakdown of glutathione, and the transportation of its amino acids back into the cell. γ-glutamyl transpeptidase (GGT) is a membrane bound enzyme involved in the transfer of glutamyl moiety, which results from the GSH breakdown, and hence allows the γ-glutamyl cycle to take place [52] (Figure 6).

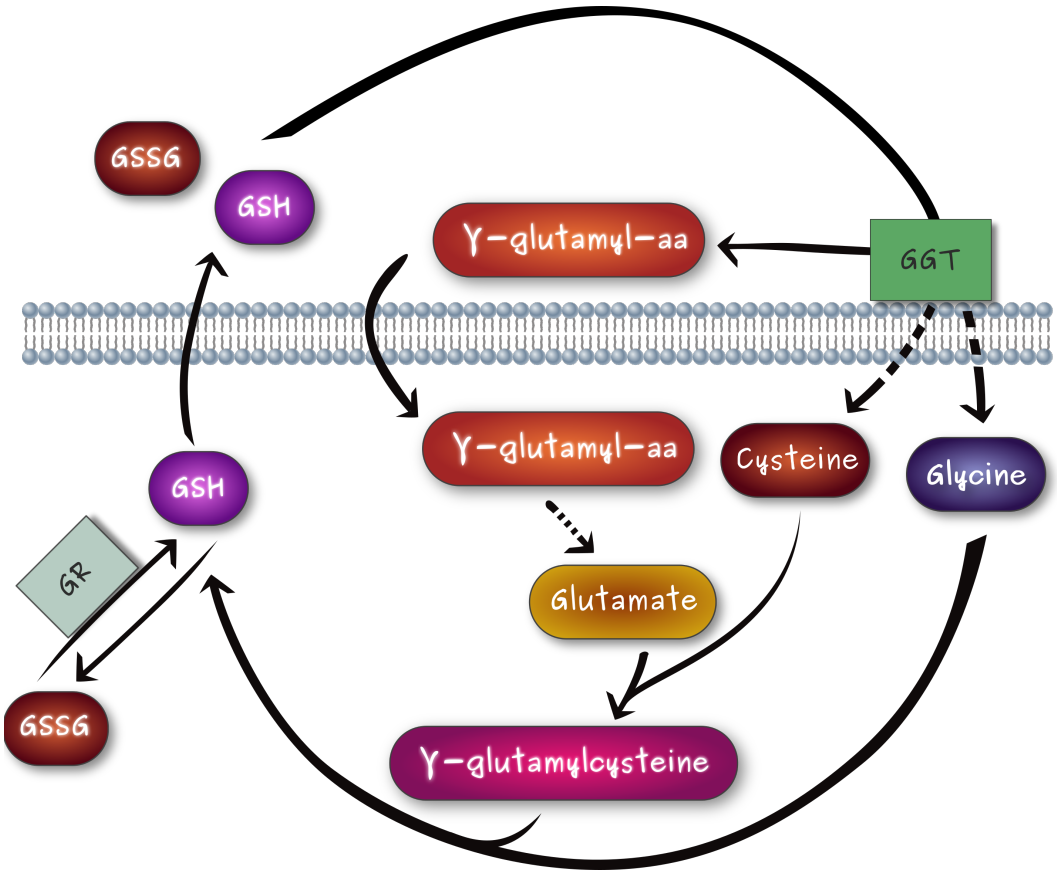

Figure 6. The γ-glutamyl cycle

aa = amino acids

1.7.3 Role of glutathione in cancer

Oxidative stress, an imbalance between production and disposal of free radicals and ROS, is a common feature of different types of cancer. Prolonged exposure of high levels of ROS, due to sustained stress, leads to perturbations of redox balance and oxidative stress. This potentially contributes to increased DNA damage or mutations and genome instability, and triggers neoplastic transformations. Enhanced ROS or oxidative stress can also stimulate cellular proliferation by inducing regulators of cell growth, proliferation and cell cycle control or by activating certain signaling pathways that contribute to tumor development. Cancer cells exhibit higher levels of basal ROS than normal cells, and increased ROS or oxidative stress plays a great role in initiation and progression of many cancers, including prostate cancer [54].

To survive in the presence of excessive oxidative stress, tumor cells counteract ROS by relying on glutathione or strategically adjusting several antioxidant enzymes. Accordingly, elevated glutathione levels can be observed in various types of tumors, and are correlated with increased proliferation and metastatic activities of malignant cells. Furthermore, the high glutathione content in tumor cells are often associated with higher levels of glutathione-related enzymes or glutathione-transporting export pumps. The expressions and activities of glutamate cysteine ligase (GCL), the key enzyme of glutathione biosynthesis, are frequently enhanced. Elevated levels and activity of γ-glutamyl transpeptidase (GGT), the essential enzyme in the γ-glutamyl cycle, have also been observed in several cancers, and are correlated with increased invasive growth [52].

Chemotherapeutic drugs, such as doxorubicin and taxol, generate high levels of ROS and oxidative stress[57,58]. As a consequence, tumor cells make use of accessible supply of glutathione or its precursor amino acids to inhibit drug-induced ROS and oxidative stress. In addition to interaction with ROS, glutathione also contributes to drug resistance by directly binding to or reacting with drugs. High glutathione levels in combination with elevated expression of glutathione-S-transferase (GST) can increase conjugation and detoxification of chemotherapeutic agents. As a consequence, tumor cells become more resistant to chemotherapy. Moreover, cells that overexpress GGT have been demonstrated to be more resistant to anti-cancer agents such as doxorubicin, cisplatin and 5-fluorouracil (5-FU) [52].

Hence, the regulation of ROS or oxidative stress has an impact on both tumor development and responses to chemotherapy.

1.8 THE TUMOR SUPPRESSOR TP53

A key player in the protection against cancer as well as in regulating responses to chemotherapeutic drugs, is the tumor suppressor gene TP53. This gene codes for the p53 protein that acts as “the guardian of the genome”. It plays an essential role in protecting us from cancer by sensing cellular stress, working to repair the damage, or triggering cell death through apoptosis.

1.8.1 p53 function and its targets

The p53 protein is widely known for its role as a DNA-binding transcription factor that activates the expression of stress response genes. Once cells sense DNA damage or other cellular insult, sensor proteins alert p53, which then leads to induction of p53's target genes that are responsible for executing the p53 response. As a result, cells undergo p53-dependent cell cycle arrest, DNA repair, or cell death (Figure 7). In that manner, p53 functions as an emergency brake to prevent transformation and proliferation of cells that carry damaged DNA or genetic lesions that lead to dysregulated growth. It may also act as a major barrier to tumor progression by preventing the accumulation of oncogenic mutations [59].

Among the most well-known p53 targets, the cell cycle inhibitor p21, also known as WAF1 or cyclin-dependent kinase inhibitor 1 (CDKN1A), was the first to be identified [60]. p21 is transcriptionally induced by p53 to regulate cell cycle progression, which predominantly leads to cell cycle arrest [61]. However, p21 can also be activated via p53-independent pathways [62]. Some other p53 target genes play key roles in the induction of apoptosis as a response to cellular stress. Upregulation of pro-apoptotic genes such as Bax, PUMA, and Noxa triggers cell death [63] (Figure 7).

Another important target of p53 is Mdm2, which is a negative regulator of p53 itself (Figure 7). Expression of the Mdm2 gene is regulated by p53 and is at the same time the key regulator of p53 protein stability. The MDM2 protein binds to p53 and targets it for ubiquitin-mediated proteolysis. p53 activation, for instance through phosphorylation at serine 15 following DNA damage, disrupts binding to MDM2. As a consequence, the p53 protein is stabilized. In this way, the interaction between p53 and MDM2 creates a negative feedback loop [61].

In addition to its critical role in DNA damage response, growth arrest and apoptosis, p53 has also been identified as an important regulator of other cellular processes, such as senescence, autophagy and metabolic pathways, including modulation of glucose uptake, dampening glycolysis and enhancement of mitochondrial respiration [64]. p53 also has the ability to regulate redox homeostasis. p53-dependent induction of p21 protects cells against oxidants by inducing the antioxidant regulator Nrf2, while targets such as Bax, PUMA and Noxa are prooxidant and promote apoptosis. p53 also has other target genes that are involved in redox regulation [65].

Furthermore, p53 is critical for the cellular response to chemotherapeutic drugs. Several chemotherapeutic agents induce DNA damage and hence cause cytotoxic effects on actively proliferating cancer cells. The cytotoxic action of these agents leads to execution of the cell death program by p53. Therefore, p53 is important for the cellular response to anti-cancer agents, and the involvement of p53 in this response suggests a mechanism through which cancer cells might develop resistance to chemotherapy [66].

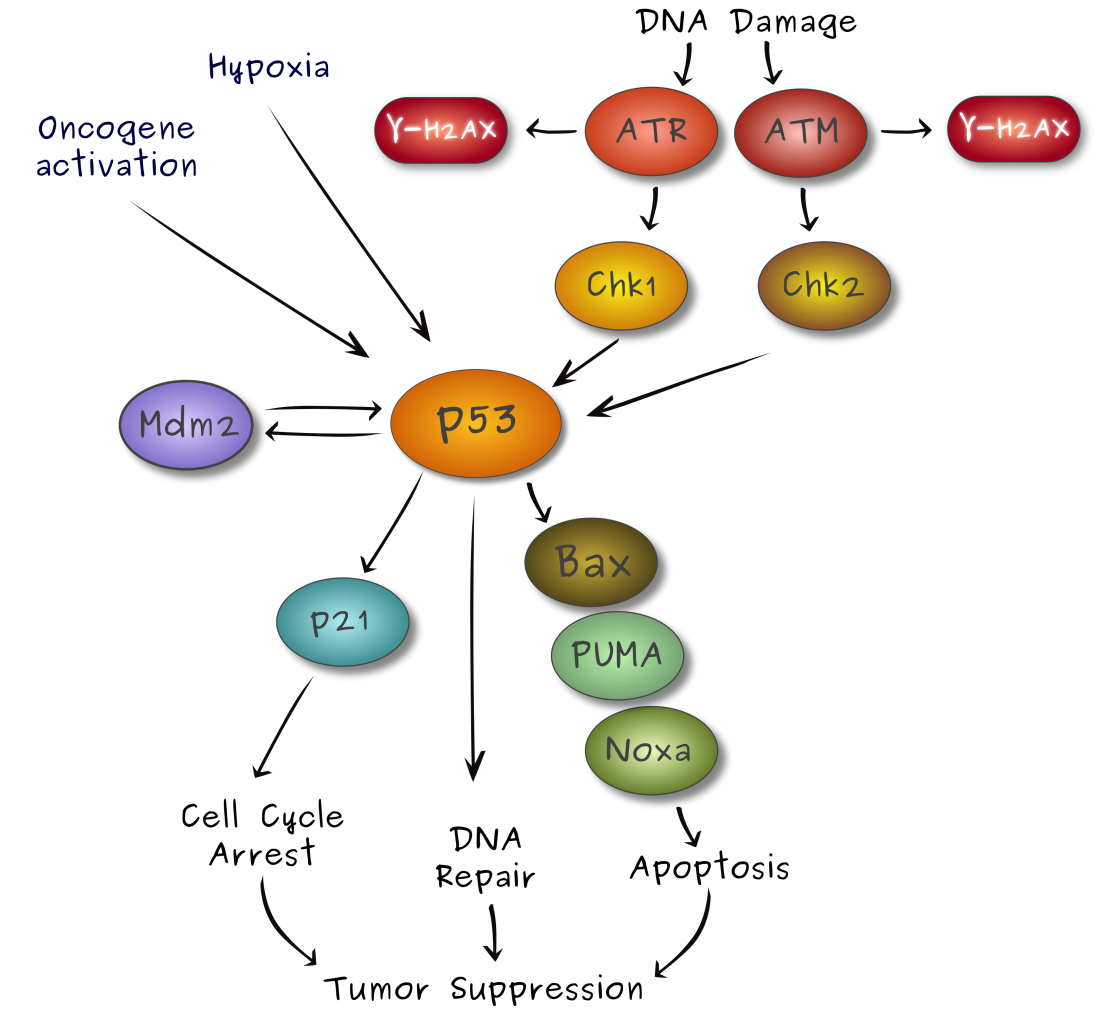

Figure 7. The p53 pathway and p53-mediated tumor suppression.

ATR = Ataxia telangiectasia and Rad3-related protein, ATM = Ataxia telangiectasia mutated protein kinase, Chk1 = Checkpoint kinase 1, Chk2 = Checkpoint kinase 2, γ-H2AX = Gamma-H2A histone family member X (phosphorylated H2AX)

1.8.2 TP53 inactivation and mutations

Given the importance of p53 in cellular stress responses and elimination of incipient tumor cells, it is not surprising that p53 is frequently downregulated or inactivated in cancer. During cancer development, cells may find many ways to repress p53 without the need to mutate the TP53 gene. Mechanisms of p53 inactivation include complex formation with viral proteins such as the human papilloma virus (HPV) E6 protein, elevated expression of MDM2, or deregulation of upstream signals, resulting in disruption of the p53 signaling pathway.

Regardless, mutations in the TP53 gene have been found in about half of human cancers [67]. In some types of cancer, such as ovarian cancer, the frequency is as high as 95% [68], while in prostate cancer it is relatively low [69,70]. The majority of TP53 alterations are missense mutations in the DNA-binding core domain [67], which results in deficient DNA binding and hence failure to activate p53 target genes.

Inactivation of p53 is a critical step during tumor evolution. Mutations in the TP53 gene essentially lead to loss of its tumor suppression functions, allowing cells to escape from apoptosis and sustain tumor growth. Besides loss of function of the protein, TP53 mutations may also endow the mutant protein with new functions. Many mutant p53 proteins acquire so called gain-of-function activities and oncogenic properties that are crucial for tumor progression. In most cases, mutant p53-carrying tumor cells accumulate high levels of functionally deficient p53 protein [67], unlike normal cells where p53 is expressed at low levels due to its short half-life [71] and only accumulates when cells experience certain stress conditions. Following recovery from cellular stress, p53 normally returns to a basal level, which is maintained by MDM2. Additionally, high levels of mutant p53 protein in tumor cells is often linked to increased resistance to anti-cancer treatments and other survival advantages [72]. Tumors that carry mutant TP53 are generally more resistant to therapy [73].

1.8.3 Rescuing the tumor suppressor p53

Since p53 mutation is common in cancer, intense efforts are being made in academia and industry to develop drugs that either inhibit the MDM2-p53 interactions, or target mutant p53 to restore its tumor suppression function [59]. However, it is a challenge to design a compound that efficiently repairs mutant p53 and restores its multiple functions. Also, mutant p53 is a heterogeneous group of proteins that includes both DNA contact and structural mutants, and therefore mutant p53 is a difficult therapeutic target. However, our lab has developed a small molecule, known as APR-246 (or PRIMA-1Met)[74-76] that is currently tested in phase III clinical trials in combination with azacitidine (a hypomethylating agent) in myelodysplastic syndrome (MDS) (a malignancy where immature blood cells in the bone marrow fail to mature). For more information regarding the clinical trials, see clinicaltrials.gov. In an earlier phase I/II clinical studies, APR-246 showed a favorable toxicity profile [77]. APR-246 is a prodrug that is converted to the active compound methylene quinuclidinone (MQ), a Michael acceptor that binds covalently to cysteines in p53, stimulating refolding to wild type conformation. This “wild-type-like” p53 can induce downstream target genes and trigger cell death [74,76,78,79] (Figure 8). Thus, APR-246/MQ restores wild type activity to mutant p53.

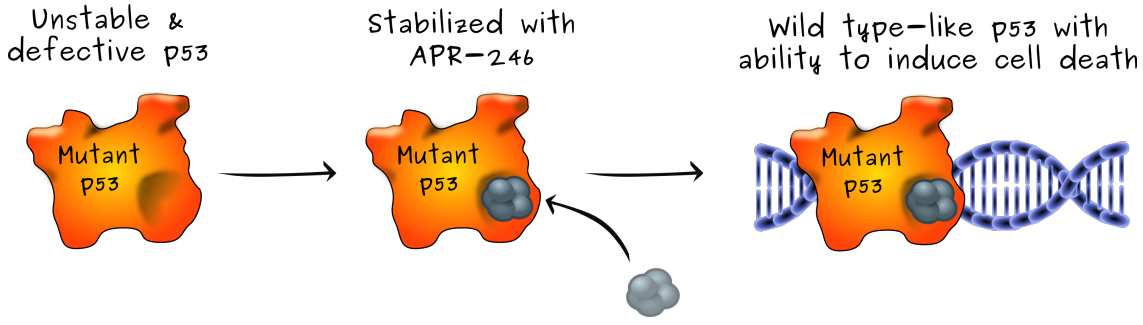

Figure 8. p53 reactivation by APR-246

1.9 CANCER TREATMENT

In order to plan treatment and predict the patient's prognosis, cancer staging is used to help determine the extent of the disease. Staging is a way to describe the size of the cancer and how far it has spread. There are two main types of staging systems, the TNM (Tumor, Node, Metastasis) system and the numbered cancer stage system. The TNM system refers to how big the initial cancer is, and whether it has spread to the lymph nodes and metastasized to other parts of the body. The number system divides cancers into stages, typically labeled from I to IV with IV being the most serious and indicating that the cancer has spread to other organs [80].

Given the complexity and multitude of behaviors for various forms of cancer, it is not always easy to decide on the most optimal treatment. Some tumors grow and spread fast while others are less aggressive. The choice of treatment for each patient depends, again, on the type and stage of cancer, as well as where it originated. Other factors such as how the treatment affects the patient's normal body functions or the patient's age and overall health are also considered.

The traditional therapies and most widely used treatment options are surgery, chemotherapy and radiation therapy (Figure 9). Surgery is used to remove solid tumors, while radiation works by breaking the cells' DNA using high-energy electromagnetic waves. Surgery and radiation are usually local treatments, aimed at the site of the tumor. Chemotherapy, on the other hand, is systemic and exposes the whole body. Furthermore, radiation and chemotherapy can be used after the surgery to kill any remaining cancer cells, or used to shrink a tumor before surgery.

Sometimes chemotherapy is the only cancer treatment needed. To date, there are more than 100 chemotherapeutic agents available. Some cancers can be treated with a single anti-cancer agent, but often several are used in a certain order or in certain combinations. Combination chemotherapy takes advantage of using drugs that work in diverse ways together to kill cancer cells more efficiently. This may help lower the risk of cancer becoming resistant to any drug. Chemotherapeutic or anti-cancer agents can be classified by their mechanisms of action. Most of them target cells at different phases of the cell cycle. Therefore, chemotherapy is most effective at killing cells that are rapidly dividing, like cancer cells.

Despite the accelerated rate of cell division usually found in cancers, which make them better targets for cytotoxic chemotherapy, the drugs do not distinguish between healthy and cancerous cells. Therefore, normal cells will be harmed along with the cancer cells. This unfortunately results in side effects that in some cases can be severe. For this reason, there is always a constant need to develop or improve drugs that can reduce potential side effects without compromising the clinical efficacy.

More recently, novel therapies such as hormone therapy, immunotherapy and targeted therapy tailored for a specific type of cancer, have been added to the therapeutic arsenal (Figure 9). These are usually adjuvant treatments and often have fewer side effects than chemotherapy. For early prostate cancer, common treatment options are surgery, also called radical prostatectomy, and radiation therapy. Radical prostatectomy is the removal of entire

prostate gland and some of the tissue around it. In cases of metastatic prostate cancer, radiation therapy along with hormone therapy, also called androgen suppression therapy, or chemotherapy may be used.

Targeted therapy refers to the use of drugs that attack cancer cells with higher selectivity than the traditional chemotherapeutic drugs, by taking advantage of differences between normal cells and cancer cells, usually specific gene alterations that drive cancer cell growth. Over the last few decades, a number of new drugs have been developed to target proteins that cancer cells depend on. A good example is imatinib or Gleevec, a drug that inhibits the oncogenic BCR-ABL kinase that is often activated in chronic myeloid leukemia (CML) [81]. APR-246 has been developed to target and reactivate mutant p53 protein. IL-6 receptor and STAT3 inhibitors aim to target cancer cells that rely on the IL-6/JAK/STAT3 signaling pathway to survive.

Uncovering genetic lesions in cancer not only provides useful information to guide treatment decisions, but also helps monitor outcome of treatment, as well as predicting relapse.

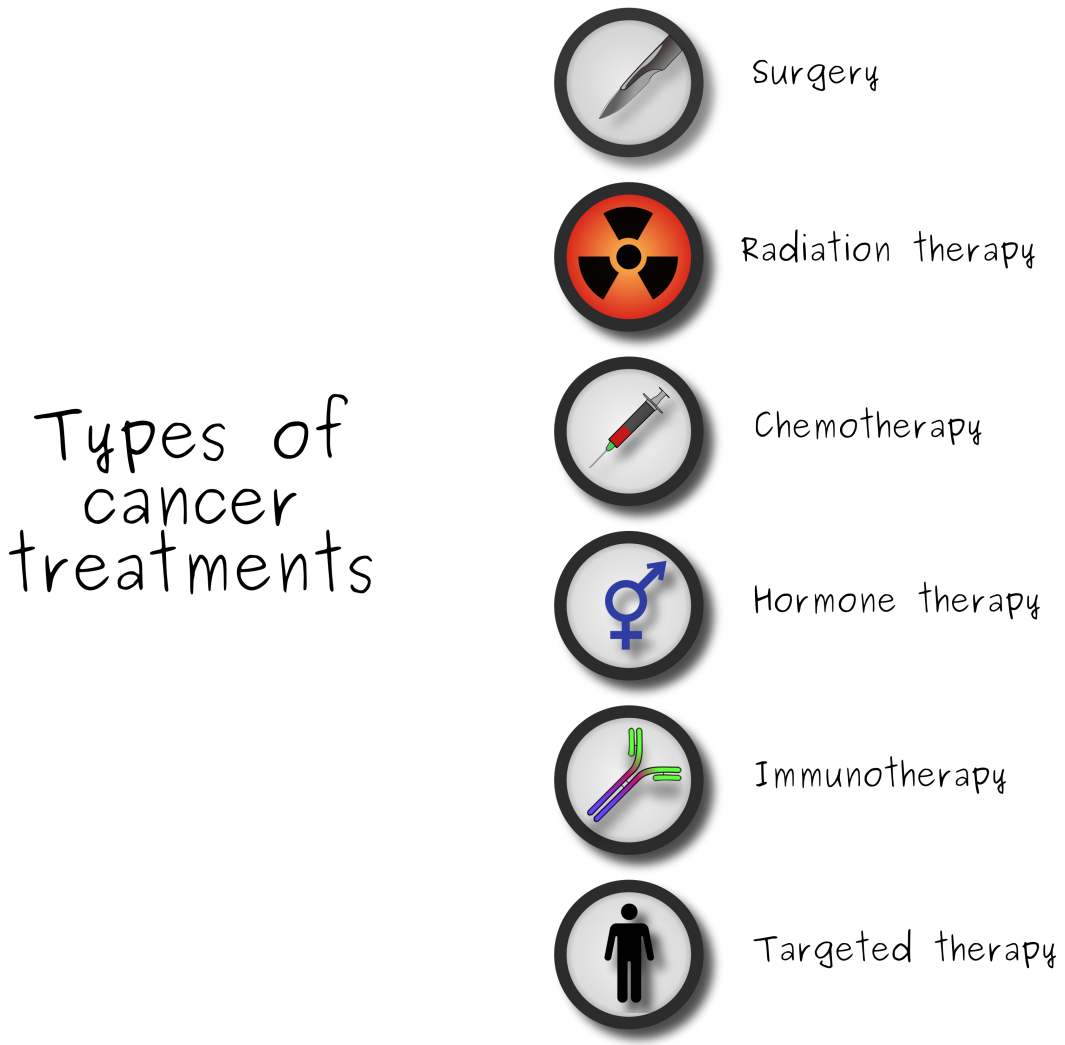

Figure 9. Cancer treatment options

1.10 CANCER DRUG RESISTANCE

Initially, most tumors respond well to therapy but tend to relapse following treatment. Development of drug resistance and relapse therefore remain as important obstacles to successful cancer therapy. Cancerous cells are persistent in finding ways to adapt and overcome therapy, making the problem very challenging. There are numerous molecular mechanisms that enable cancer cells to evolve therapy resistance.

1.10.1 Interactions with the surrounding microenvironment

The reappearance of tumor cells after the insult of therapy, even if the number of cells is very small, can unfortunately seed the growth of a new tumor. Due to the presence of multiple clones in a tumor, small subpopulations of cells may survive treatment and then be further selected, resulting eventually in tumor relapse [82].

Moreover, cancer cells may be shielded by interacting with their microenvironment. The tumor microenvironment provides the aid they need and protects them from therapy, which may consequently lead to less effective treatment. One mechanism by which the tumor microenvironment confers drug resistance is soluble factors. Hepatocyte growth factor (HGF), often overexpressed by CAFs, has been shown to play an essential role in the development of drug resistance to different kinase and growth factor receptor inhibitors. For instance, HGF contributes to resistance to serine/threonine-protein kinase B-Raf inhibitor used to treat melanoma, human epidermal growth factor receptor 2 (HER2) inhibitor used as a treatment for HER2 positive breast cancer, and epidermal growth factor receptor (EGFR) inhibitor for colorectal cancer [83]. C-C motif chemokine ligand 2 (CCL2), which can be induced by CAFs, has also been reported to confer resistance to paclitaxel (also known as taxol) and docetaxel in prostate cancer [83]. Another study demonstrated that prostate fibroblasts are able to attenuate the effects of chemotherapy through secretion of WNT16B and thus promote tumor cell survival [84].

The interstitial fluid pressure, which is normally regulated through interactions between extracellular matrix (ECM) molecules and stromal cells, is abnormal in tumor tissues. Many solid tumors show an increased interstitial fluid pressure when compared to their surroundings, due to features such as blood and lymph vessel abnormalities, increased number of fibroblasts and a dense network of collagen fibers in the ECM [15]. Dense and stiff ECM, as well as high interstitial fluid pressure caused by CAFs, are two critical barriers for cancer therapy. These two factors decrease the amount of drug that reaches the tumor cells and thus limit the efficiency of drug penetration, resulting in poor anti-tumor therapy response [15,85].

1.10.2 Alterations within the cancer cells

Cancer cells are masters of adaptation. In order to elude treatment, they are able to alter drug uptake, efflux, and detoxification to limit the intracellular concentration of various therapeutic compounds. The drug resistance can result from modification of the activity or expression of surface receptors and transporters. For instance, increased expression of the ATP binding cassette (ABC) membrane transporters results in enhanced drug efflux. Among the known ABC transporters, multidrug resistance 1 (MDR1), multidrug resistance-

associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) have frequently been correlated with cancer chemoresistance [82].

Cancer cells are also able to circumvent the effects of the drugs by preventing anti-cancer drugs from undergoing metabolic activation, or inactivating them through conjugation to glutathione. The GSH-conjugated compounds become substrates for ABC transporters, such as MRP1, which increases the export of drugs from cells [82]. Glutathione can also stimulate the activity of MRP1 and further facilitate drug transport [86]. In addition, MRP1 plays a role in redox regulation and the cellular efflux of GSH and GSSG, influencing the susceptibility of cancer cells to oxidative stress [87].

Furthermore, the use of drugs or inhibitors that target one specific protein alone will likely lead to selection for mutations in the target protein that make the drug ineffective after a long-term treatment. Alternatively, cancer cells discover parallel pathways that support their survival, which then leads to amplification of alternative oncogenes and activation of other survival pathways [82].

Because of the flexibility of cancer cells and the ongoing selection for enhanced cell survival in a developing tumor, cancer cells can often find ways to evade programmed cell death induced by therapy. Apoptotic pathways are frequently disrupted in tumor cells [82]. Indeed, p53 that plays a critical role in inducing apoptosis in response to treatment is mutated and linked to chemoresistance in many cancers [88]. For instance, TP53 mutations have been correlated with acquired resistance to doxorubicin in patients with advanced breast cancer [89]. A very recent study showed that a mutant TP53 RNA expression signature correlates with reduced survival in eleven cancer types [90]. In addition to mutations in TP53 itself, p53 activity may indirectly be affected due to dysregulation of factors upstream of p53, e.g. proteins that are involved in the DNA damage response [82] (Figure 7). Moreover, p53 function can also be disrupted due to alterations of negative p53 regulators such as MDM2, which results in inhibition of normal p53 function and poor treatment outcome [88].

2 AIMS OF THE THESIS

The overall aim of this thesis was to elucidate pathways or mechanisms of CAF-mediated drug resistance in cancer, and to investigate alternative targeted therapy.

The specific aims of the papers were:

Paper I: To investigate the impact of human prostate CAFs on chemotherapy resistance and prostate cancer cell survival, as well as the mechanism underlying this effect.

Paper II: To examine the role of CAF-derived soluble factors, particularly IL-6, on drug resistance and drug-induced p53 response in prostate cancer cells.

Paper III: To further clarify the mechanism of action of the mutant p53-reactivating compound APR-246, and assess whether inhibition of MRP1 synergizes with APR-246.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Human cancer-associated fibroblasts enhance glutathione levels and antagonize drug-induced prostate cancer cell death.

Cells of the tumor microenvironment have in recent years become increasingly recognized as critical players in tumor development and progression, as well affecting therapeutic outcomes. CAFs are the most abundant cell type in the tumor microenvironment and prostate cancer is known to contain high numbers of these cells. Drug resistance in prostate cancer also remains a challenge to successful treatment. Therefore, I have investigated the role of prostatic CAFs in conferring drug resistance and promoting prostate cancer cell survival. In particular, I have examined how CAFs affect the response to different chemotherapeutic agents in prostate tumor cells carrying wild type TP53.

Prostate cancer cells were co-cultured with CAFs and treated with different DNA-damaging agents, including doxorubicin, taxol and mitomycin C. In all three cases, I observed an increase in tumor cell survival and attenuation of the drug-induced p53 response. Medium conditioned by CAFs showed comparable effects on tumor cell death and p53 induction, indicating that CAFs modulated the drug sensitivity through one or several soluble factors.

Cancer cells that were co-cultured with CAF-conditioned medium also demonstrated lower levels of DNA damage, drug accumulation and ROS after exposure to doxorubicin, as compared to cancer cells grown in fresh medium. In line with this, I observed enhanced intracellular levels of glutathione in cancer cells in the presence of CAF-conditioned medium, as well as elevated levels of oxidized glutathione in CAF-conditioned medium. Interestingly, both glutathione and its precursor cystine were able to decrease doxorubicin content in cancer cells, although to a different extent. All of this together argues that CAFs protect cancer cells by decreasing sensitivity to anti-tumor agents through glutathione, which modulates the redox balance and drug-induced ROS levels in cancer cells, and possibly also interferes with the amount of drug that reaches the cancer cells.

Whether decreased doxorubicin accumulation was a result of reduced drug influx, increased efflux or other factors have not been thoroughly investigated in this study. A rescue experiment with an MRP1 inhibitor was performed in the attempt of finding out whether the MRP1 transporter was involved, given that doxorubicin, taxol and glutathione are substrates for this pump. However, there was no difference in the amount of doxorubicin accumulated in the tumor cells cultured with CAF-conditioned medium or fresh medium, and no conclusions were drawn based on those results.

In summary, I have identified glutathione secreted by CAFs as a critical component in the tumor microenvironment, which has the potency to reduce the chemotherapeutic drug response in prostate cancer cells. CAF-derived glutathione is taken up by cancer cells in order to shelter them from chemotherapeutic agents, as well as drug-induced damage and stress. Consequently, the tumor cells are able to survive the treatment.

From this study, I gained a better understanding of how CAFs mediate protection of cancer cells against chemotherapeutic drugs and revealed a possible mechanism underlying this effect. The knowledge gained may provide opportunities to improve cancer treatment by targeting CAFs or interfering with interaction between stroma and tumor cells. The strategy of inhibiting glutathione production by CAFs or blocking uptake and resynthesis of glutathione by cancer cells, in combination with other chemotherapeutic drugs, may allow more efficient elimination of tumor cells.

3.2 PAPER II

Interleukin-6 derived from cancer-associated fibroblasts attenuates the p53 response to doxorubicin in prostate cancer cells.

In the process of identifying CAF-derived soluble factors that are responsible for increased drug resistance in the previous study, I also used a cytokine array approach and was able to detect a number of cytokines and growth factors in the CAF-conditioned medium. Several of these soluble factors were secreted only by CAFs and not by cancer cells. The fresh medium also did not contain these components. Among the identified secreted factors, I tested our hypothesis using recombinant HGF, IL-6 and osteoprotegerin (OPG), a soluble member of the tumor necrosis factor (TNF) receptor superfamily. Only IL-6 showed capacity to enhance cell survival and reduce the p53 response to doxorubicin in prostate cancer cells, without affecting drug accumulation. The attenuation of p53 induction was associated with increased MDM2 mRNA levels, Mdm2 protein binding to p53, and p53 ubiquitination. These observations indicated that the p53 protein was targeted for degradation, a process mediated by Mdm2. Thus, IL-6 inhibits p53 accumulation and thus function, which is important for the response to chemotherapeutic drugs.

To examine the mechanism through which IL-6 might exert its action on reducing drug efficacy, I tested several inhibitors, including those against JAK, STAT3, PI3K and Akt, in order to block these factors downstream of IL-6 in the IL-6 signaling pathways. Through this approach, I could identify JAK and STAT3 as essential players in drug resistance mediated by IL-6.

Furthermore, analyses of publicly available datasets also supported the findings above and revealed that amplification of the IL-6 receptor (IL-6R), STAT3 and MDM2 genes was more common in prostate tumors with unaltered TP53 gene than in tumors with altered TP53. The amplification of these genes also seemed to be more frequent in metastatic castration-resistant prostate cancer. The datasets further revealed higher IL-6R and MDM2 mRNA expression levels in prostate tumors with unaltered TP53 compared to tumors with putative driver TP53 mutation. IL-6R, JAK and STAT3 mRNA levels also correlated with MDM2 mRNA levels in prostate tumors carrying unaltered TP53. All these findings suggest that there is selective pressure to inactivate p53 via IL-6, JAK, STAT3 and MDM2 in TP53 wild type prostate tumors, and that MDM2 may be upregulated by the IL-6/JAK/STAT3 pathway. The higher prevalence of IL-6R, STAT3 and MDM2 amplification in metastatic prostate tumors may also explain drug resistance in advanced and aggressive diseases.

Altogether, this study suggests another possible mechanism by which CAFs may confer drug resistance to tumor cells, and highlights IL-6 as a component in the tumor microenvironment with the ability to attenuate the drug-induced p53 response through the JAK/STAT3 signaling pathway. This negatively affects the treatment outcome.

The CAF-mediated protective effect on cancer cell survival may be circumvented by targeting IL-6, or block the crosstalk between CAFs and cancer cells. Hence, the combination of targeted treatment with chemotherapeutic drugs may lead to improved and more effective cancer therapy. Several agents targeting the IL-6/JAK/STAT3 signaling pathways are being tested in clinical trials and evaluated in patients with different types of tumors, while some are currently in development.

3.3 PAPER III

A thiol-bound drug reservoir enhances APR-246-induced mutant p53 tumor cell death

The mutant p53-reactivating compound APR-246 is converted to the active product MQ, which readily reacts with cellular thiols [74,76]. MQ also binds to cysteine residues in p53 [76,79]. Previous studies have demonstrated that APR-246 or MQ can deplete glutathione and inhibit the antioxidant systems [76,91-93]. Tumor cells that exhibit high levels of glutathione are found to be more resistant to treatment with APR-246 [94]. Endogenous glutathione, as well as drugs conjugated to glutathione, are exported through the efflux pump MRP1 [95]. Given the roles of MRP1 in redox regulation and transporting glutathione-conjugated drugs, as well as association with drug resistance, we hypothesized that inhibition of MRP1 using the inhibitor MK-571 and trapping APR-246/MQ that conjugates to glutathione inside tumor cells would enhance therapeutic efficacy of APR-246.

We tested the combination treatment of APR-246 with the MRP1 inhibitor MK-571 in several tumor cell lines and found that the combination treatment resulted in synergistic tumor cell death with a more pronounced effect in cells carrying mutant TP53. Combining APR-246 with Reversan, another MRP1 inhibitor, or with siRNAs against MRP1, gave similar results. We further confirmed our findings in oesophageal cancer cell xenograft mouse models and observed enhanced APR-246 anti-tumor activity in combination with MK-571. Both *in vitro* and *in vivo* experiments revealed that MRP1 blockade significantly enhanced APR-246-induced mutant p53 cancer cell death.

The observed synergy was associated with increased intracellular drug accumulation. We analyzed drug content, specifically ^{14}C accumulation, in various cell lines using radiolabelled ^{14}C-APR-246, where ^{14}C is retained in MQ, in combination with MK-571, Reversan or MRP1 siRNAs. We found that MQ bound to glutathione (GS-MQ) was accumulated and also showed that the binding of MQ to glutathione is reversible. Thus, altogether these results reveal that MQ is exported via MRP1 and blocking MRP1 leads to the accumulation of MQ inside cells. GS-MQ is formed within cells and is remained trapped intracellularly upon MRP1 inhibition. Due to the reversibility of the conjugation formation, GS-MQ may serve as

a drug reservoir, increasing the availability of MQ for mutant p53 reactivation. This enhances sensitivity of mutant TP53-carrying cancer cells to APR-246.

Furthermore, the cellular redox status was altered by the combination treatment. We detected an increase in intracellular cystine and cysteine, key building blocks for glutathione synthesis, as well as an elevated expression of xCT, the transporter of cystine, upon MRP1 inhibition by MK-571. After treatment with APR-246 and MK-571, the expression of xCT increased and the intracellular levels of GSH, GSSG and cysteine were decreased. Blocking xCT with the xCT inhibitor sulfasalazine (SSZ) or using siRNAs also resulted in increased ^{14}C accumulation after ^{14}C-APR-246 treatment, and increased APR-246-induced cell death. These results indicate that glutathione and cysteine availability determines drug accumulation and sensitivity to APR-246. The presence of mutant p53, cellular redox state, and drug accumulation together affect the cell death-inducing activity of APR-246.

Overall, inhibition of MRP1 with MK-571 blocks drug export and leads to alterations in the cellular redox status, which then enhance the cell-death inducing activity of APR-246. This further verifies that cancer cells rely on glutathione to make themselves less sensitive to cancer therapy and drug-induced stress. Our results also suggest that combination treatment with APR-246 and inhibitors of MRP1 could improve clinical efficacy of the mutant p53-reactivating compound APR-246.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

Cancer remains one of the leading causes of death globally. Despite immense amount of research in cancer biology and cancer drug discovery, many tumors still have poor prognosis. Therapy resistance is one of the major problems in anti-cancer therapy, and contributes to the high mortality rates. For this reason, it is essential to uncover molecular mechanisms governing cancer drug resistance. Development of novel strategies and targeted therapies to improve the outcome of treatment is critically needed. An increased understanding of the diverse molecular mechanisms that cancer cells depend on may provide opportunities for more efficient therapy by targeting disease-specific mechanisms or several pathways at the same time. Combination of targeted therapy with chemotherapy or other therapies may lead to more efficient treatment of cancer.

CAFs, essential components of the tumor microenvironment, can protect cancer cells from therapy through several mechanisms. Therefore, they represent a potential therapeutic target for the treatment of cancer. In the **paper I** and **II**, chemoresistance-associated molecules, produced by CAFs, have been identified. My studies revealed that glutathione and IL-6 could promote chemoresistance in prostate cancer cells carrying wild type p53 through different mechanisms. Glutathione shields cancer cells from drug-induced oxidative stress and DNA damage, as it also decreased drug accumulation, while IL-6 attenuates the drug-induced p53 response through STAT3 and MDM2. Depletion of these molecules, or targeting the interaction between stroma and cancer cells, may therefore decrease or prevent therapy resistance in cancer. Thus, the co-targeting of cancer cells and their microenvironment is likely to be a fruitful strategy for improved cancer treatment.

Other soluble factors than those discussed in **paper I** and **II** can presumably increase drug resistance, given that CAFs secrete a vast repertoire of soluble factors, as shown in **paper II**. Thus, the identification of novel molecules linked to resistance and poor prognosis may provide novel clinical prognostic markers and open novel avenues for more efficient therapy for different types of cancer.

Accordingly, the findings from **paper I** and **II** are in line with other studies depicting a critical role of the tumor microenvironment, specifically CAFs, in cancer progression and development. The papers thus confirm that CAFs can have a significant impact on the therapeutic response and extend current understanding by identifying specific molecular mechanisms.

Paper III demonstrates that inhibition of the MRP1 drug efflux pump can enhance sensitivity of mutant TP53-carrying cancer cells to APR-246, as combination treatment of APR-246 with MRP1 inhibitors resulted in synergistic cancer cell death. This was associated with altered cellular redox status and increased intracellular MQ, the active product of APR-246, conjugated to glutathione. The reversibility of MQ conjugation may lead to increased availability of MQ for targeting mutant p53. Our findings revealed that several players are involved in determining sensitivity to APR-246 treatment. The presence of mutant p53, redox state in the cells and drug accumulation together affect the cell death-inducing activity of APR-246, which could be potentiated by MRP1 inhibition. Hence, this study also highlights

the impact of the cellular redox status and glutathione content on the cancer cell survival and response to mutant p53-targeted therapy.

The studies in this thesis have revealed several putative drug targets, including glutathione and redox signaling molecules, the IL-6/JAK/STAT3 signaling pathway, and the MRP1 transporter. Blocking these resistance-promoting pathways may greatly enhance the efficacy of chemotherapy and other types of therapy. High expressions of enzymes involved in glutathione synthesis, IL-6, STAT3, MDM2, mutant p53, and MRP1 in tumors may also represent useful predictive markers of drug resistance. Since non-cancerous cells of the tumor microenvironment are genetically more stable than tumor cells, they represent a favorable therapeutic target with lower risk of drug resistance. Hence, targeting the tumor microenvironment has huge potential for future cancer therapy.

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